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Expansins and Internodal Growth of Deepwater Rice[1,2]

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The distribution and activity of the cell wall-loosening protein expansin is correlated with internodal growth in deepwater rice (*Oryza sativa* L.). Acid-induced extension of native cell walls and reconstituted extension of boiled cell walls were confined to the growing region of the internode, i.e. to the intercalary meristem (IM) and the elongation zone. Immunolocalization by tissue printing and immunoblot analysis, using antibody against cucumber expansin 29 as a probe, confirmed that rice expansin occurred primarily in the IM and elongation zone. Rice expansin was localized mainly around the vascular bundles at the base of the IM and along the inner epidermal cell layer surrounding the internodal cavity. Submergence greatly promoted the growth of rice internodes, and cell walls of submerged internodes extended much more in response to acidification than did the cell walls of air-grown internodes. Susceptibility of cell walls to added expansin was also increased in submerged internodes, and analysis by immunoblotting showed that cell walls of submerged internodes contained more expansin than did cell walls of air-grown internodes. Based on these data, we propose that expansin is involved in mediating rapid internodal elongation in submerged deepwater rice internodes.

Internodes of deepwater rice (*Oryza sativa* L.) elongate at a fast rate in response to submergence (Vergara et al., 1976). This rapid internodal growth is driven by cell division and cell elongation. Because of the increased number and length of cells in submerged internodes, the EZ expands from 5 to 25 mm (Bleecker et al., 1986). Previous experiments have shown that the cell walls of rapidly elongating internodes maintain high extensibility (Kutschera and Kende, 1988). Such wall extensibility is thought to result from the action of wall-loosening factors (for a review, see Cosgrove, 1993). Recently, a new class of cell wall proteins, the expansins, have been discovered that have properties of wall-loosening factors (McQueen-Mason et al., 1992; for reviews, see McQueen-Mason, 1995; Cosgrove, 1996). They mediate long-term extension (creep) of isolated cell walls that are subjected to tension in acid solution. Expansins do not have detectable hydrolytic enzyme activity but appear to act by disrupting hydrogen bonds between cellulose and other wall polymers (McQueen-Mason and Cosgrove, 1994). Acid-induced creep depends not only on the presence of expansin in the cell wall but also on the susceptibility of the wall to expansin (Cosgrove and Li, 1993; McQueen-Mason, 1995).

We have recently characterized expansins from the internodes of deepwater rice (Cho and Kende, 1997). Here we report on the correlation between the elongation of different developmental regions of the rice internode, cell wall extensibility in acid buffers, the localization of expansin, and the susceptibility of isolated walls to added expansin. Our results support the hypothesis that expansins play a role in mediating elongation of submerged rice internodes.

MATERIALS AND METHODS

Rice plants (*Oryza sativa* L. cv Pin Gaew 56) were grown as described previously (Stünzi and Kende, 1989). Twenty-centimeter-long stem sections containing the uppermost internode were excised from 11- to 13-week-old plants according to Raskin and Kende (1984). For treatments in air, 15 to 20 sections were incubated in distilled water so that the water level remained below the basal node. For submergence, the sections, with a weight attached, were completely immersed in 2.5-L, 60-cm-deep Plexiglas cylinders containing distilled water (Kutschera and Kende, 1988). They were incubated for 2 d at 27°C under continuous light (cool-white fluorescent tubes, 53 μmol s^{-1} m^{-2}; General Electric).

Measurement of Cell Wall Extension

Cell wall extension was measured according to Cosgrove (1989), as modified for deepwater rice internodes (Cho and Kende, 1997). Data are means ± SE of three to five measurements.

Extraction of Cell Wall Proteins and Dry Weight Determination

Preparation of cell walls and extraction of cell wall proteins were performed according to McQueen-Mason et al. (1992), as adapted for rice internodes (Cho and Kende, 1997). For determining cell wall dry weight, salt-extracted cell walls were treated with 2 mg mL^{-1} protease from *Streptomyces griseus* (Sigma) at pH 7.0 and 37°C for 20 h. The cell walls were washed with water and 70% ethanol,

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Abbreviations: CuEx29, cucumber expansin 29; DZ, differentiation zone; EZ, elongation zone; IM, intercalary meristem.

dried at 85°C for 24 h, and weighed (Cosgrove and Li, 1993).

Immunoblot Analysis

Immunoblotting of rice expansins using the CuEx29 antibody as a probe (Li et al., 1993) was as described previously (Cho and Kende, 1997). Protein was measured using the bicinchoninic acid protein assay reagent (Pierce) with BSA as a standard.

Tissue Printing

Tissue-print immunoblotting was performed as described originally by Cassab and Varner (1987) and in more detail by Cassab (1992) for the cell wall protein, extensin. Because extensins are tightly bound to the cell wall, they are extracted with solutions containing high concentrations of salt. To successfully transfer extensin to nitrocellulose membranes by tissue printing, Cassab and Varner (1987) presoaked the nitrocellulose in 0.2 M $CaCl_2$. We followed the same procedure for expansin. To verify the applicability of this method, we established that similar results were obtained when expansin was blotted onto nitrocellulose membranes that had been presoaked either in 1 M NaCl, which is used to extract expansin from the cell wall, or in 0.2% SDS. Cross- or longitudinal sections of 0.5 mm thickness were cut with a one-edged razor blade from rice internodes. After gentle blotting on tissue paper, the sections were carefully transferred onto a nitrocellulose membrane lying on top of a sheet of photocopy paper and six layers of Whatman 1MM filter paper. After placing four layers of tissue paper on the sections, they were pressed gently and evenly for 20 s with a finger. The membrane was dried, and expansin was detected with the CuEx29 antibody, as described for immunoblotting (Cho and Kende, 1997). Preimmune serum (1:1000 dilution) was used as the primary antibody for the control. To determine the protein distribution on tissue prints, the membranes were gently shaken in a Ponceau S solution (1:10 dilution, Sigma) for 2 min and destained in water.

RESULTS

Localization of Rice Expansin by Tissue Printing

We examined the distribution of rice expansin in the uppermost rice internode by tissue printing onto nitrocellulose membranes that had been presoaked in 0.2 M $CaCl_2$ (Cassab and Varner, 1987; Cassab, 1992). Because similar results were obtained when membranes had been presoaked in 1 M NaCl, the expansin thus transferred is considered a salt-extractable cell wall protein. Earlier, we had established by immunogold labeling at the electron microscope level that expansin is almost exclusively localized in the cell wall (Hoffmann-Benning, 1993; Hoffmann-Benning et al., 1994). Tissue-print immunoblots of rice internodes using the CuEx29 antibody showed three distinct features of expansin localization (Fig. 1A). First, rice expansins were concentrated within a 1-cm region at the base of the internode. The distribution of expansin followed a gradient, with the highest level being detected in the IM and declining levels in the EZ above it. In the DZ the expansin signal was barely detectable. Tissue prints of the node just below the IM did not show any expansin signal. Second, expansins were localized at a high density around the developing vascular bundles at the very base of the IM but disappeared rapidly in the region above it. Third, a strong expansin signal was evident along the inner epidermal cell layer that surrounds the internodal cavity. The localization of expansin in the inner epidermis was also limited to the IM and EZ, as seen in both cross- and longitudinal sections.

In contrast to the above results using the CuEx29 antibody, probing tissue prints with the preimmune serum did not show any specific localization of a signal (Fig. 1B). The distribution of total cell proteins along the internode was determined on tissue prints using Ponceau S staining (Fig. 1C). There appeared to be somewhat more protein in the region of the IM than in the DZ. This may reflect the compact tissue structure and/or the higher metabolic activity in the IM and the development of air spaces in the DZ. Ponceau S staining indicated a high concentration of protein inside the vascular bundles, whereas the insides of the vascular bundles on immunoblots were very weakly stained (compare Fig. 1A with 1C). Staining of tissue prints with Ponceau S showed as high a level of protein in the node below the IM as in the IM itself.

Tissue Localization of Cell Wall Extensibility

We investigated whether extensibility of isolated cell walls correlated with the distribution of expansin along the internode, as seen on the tissue prints. Figure 2A shows acid-induced extension of 1-cm-long cell wall specimens from regions of the internode containing the IM and the EZ, the EZ and a small portion of the DZ, and the DZ. Extensibility of native cell walls was highest in the IM and the EZ. It was 27% lower in the 1-cm region, which included the EZ just above the IM and approximately 0.2 cm of the DZ; wall extensibility was low in the DZ. Figure 2B shows the susceptibility of the heat-inactivated cell walls from these same internodal regions to the added wall-protein preparation containing expansin. The pattern of wall extensibility observed in these reconstitution experiments was very similar to that found in native cell walls. The cell walls from the IM and EZ were much more responsive to added expansins than were the cell walls from the DZ.

Correlation of Growth, Wall Extensibility, and Expansin Content

Figure 3 shows the relationship between internodal growth induced by submergence and acid extensibility of cell walls. Submerged internodes elongated 5 cm over 2 d, whereas air-grown internodes grew about 0.3 cm during the same period (Fig. 3A). Acid-induced extension was much higher in the cell walls of submerged internodes than in the walls of air-grown internodes (Fig. 3B).

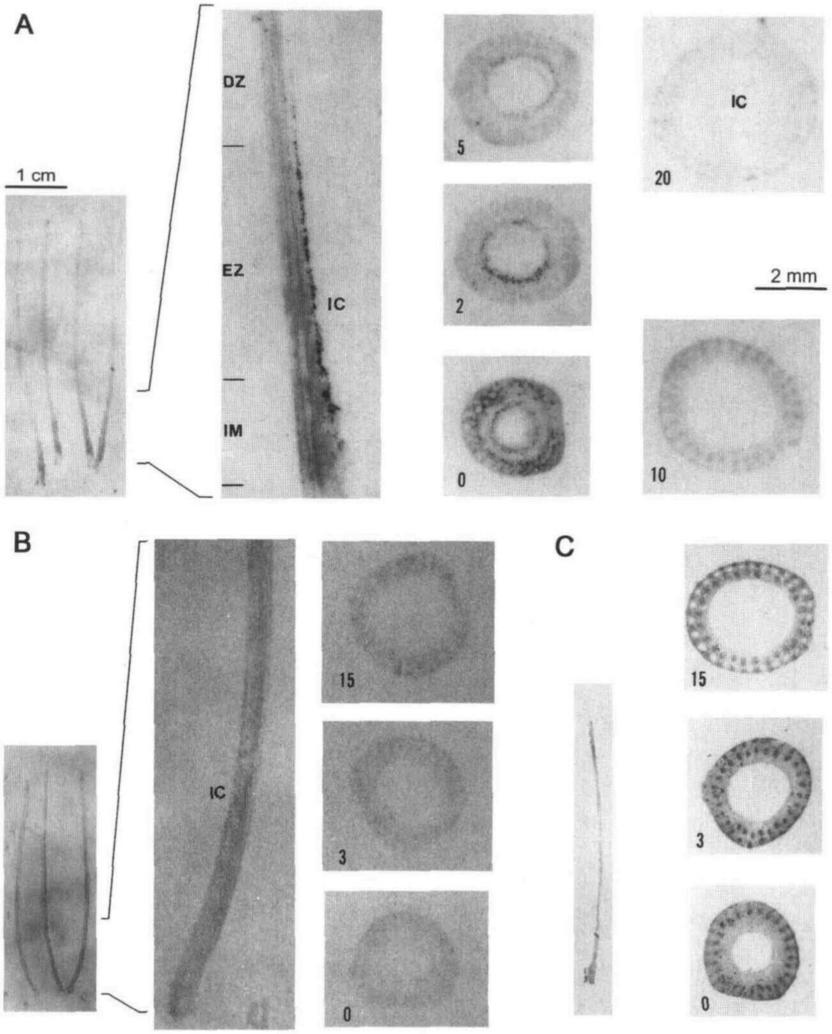

Figure 1. Localization of expansin in the uppermost rice internode using tissue printing. A, Immunoblotting with the CuEx29 antibody (1:2000 dilution) as the primary antibody. IC, Internodal cavity. B, Immunoblotting with preimmune serum (1:1000 dilution) as the primary antibody. C, Staining of proteins with Ponceau S. The numbers denote the distance in millimeters above the basal node.

We used immunoblotting with the CuEx29 antibody as a probe to determine the salt-extractable expansin content in different developmental regions of air-grown and submerged internodes. The immunoblot in Figure 4A shows expansin content per unit of cell wall dry weight. The cell walls from the basal 1-cm region of internodes that had been submerged for 2 d contained much more expansin than did the cell walls from the corresponding region of internodes that had been incubated in air for 2 d, and also more than the cell walls from the same internodal region at 0 time. The expansin content was decreased greatly in the cell walls of the older second and third 1-cm regions in all treatments. For the immunoblot in Figure 4B, the same amount of cell wall protein was loaded in each lane. In contrast to the expansin content per cell wall mass (Fig. 4A), the amount of expansin per total salt-extractable cell wall protein remained constant in the regions 0 to 1 cm and 1 to 2 cm above the node in both air-grown and submerged internodes. However, there was a decrease in the ratio of expansin to salt-extractable cell wall protein in the 2- to 3-cm region of submerged internodes and, to a lesser degree, of air-grown internodes (Fig. 4B).

Protein Content and Mass of Internodal Cell Walls

Since expansin levels have to be related to cell wall parameters, we determined the fresh weight, dry weight, and protein content of internodal cell walls in the three 1-cm regions above the node at the start of the experiment (0 time) and after 2 d of submergence or incubation in air. The fresh weight of the cell wall remained nearly constant in air but declined somewhat in submerged internodes (Fig. 5A). The dry weight of the wall increased with distance from the node in air but decreased substantially with submergence (Fig. 5B). The content of salt-extractable cell wall proteins was much higher in the basal 1-cm region of the internode than in the older regions above it, both in absolute terms (Fig. 5C) and on a dry weight basis (Fig. 5D).

Effect of Submergence on Cell Wall Extensibility

Figure 6 shows the acid-induced extension of native and reconstituted cell wall preparations from air-grown and submerged internodes. The basal 1-cm region immediately

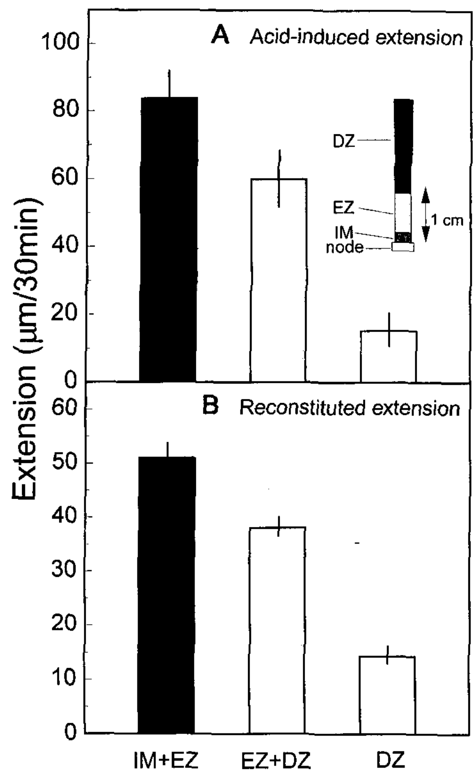

Figure 2. Cell wall extensibility of different developmental regions of rice internodes. A, Acid-induced cell wall extension. Sections 1.5 cm in length were excised from the uppermost internode, frozen, abraded, thawed, and pressed between filter paper. Segments of 1.5-mm width containing the IM and EZ, the EZ and part of the DZ, and the DZ without IM and EZ were fastened between two clamps, 1 cm apart, and were placed in an extensometer. A constant load of 10 g was applied. After pretreatment with 50 mM Hepes/Tris buffer, pH 6.8, for 20 min, the solution was changed to 50 mM sodium acetate buffer, pH 4.5. B, Reconstituted cell wall extension. Cell wall specimens prepared as described above were boiled for 1 min in water and clamped in an extensometer. After pretreatment with 50 mM sodium acetate buffer, pH 4.5, for 20 min, the solution was changed to the same buffer containing cucumber cell wall proteins (0.5 mg mL^{-1}). Cell wall extension was calculated from the change in length of the segments over a 30-min period.

above the node contained the IM and the EZ. The other 1-cm regions represented older tissue. In air-grown internodes they consisted of the DZ; in submerged internodes, the EZ extended over a longer distance (approximately 3 cm) and was followed by the DZ. Acid-induced wall extensibility was highest in the basal 1-cm region of the submerged internodes, decreased in the next 1-cm zone by one-half, and continued to decline with distance from the node (Fig. 6A). In contrast, wall extensibility of air-grown internodes was confined to the basal 1-cm region above the node and was 50% lower than in the corresponding region of submerged internodes. In boiled cell wall preparations from the same internodal regions, the pattern of reconstituted cell wall extensibility resembled that of native cell walls in both submerged and air-grown internodes with one significant difference: In submerged internodes the decrease in reconstituted wall extensibility over the first 3 cm above the node was much more gradual than the decrease in wall extensibility of native walls. This may indicate that acid-induced wall extensibility in native walls is limited more by the level of endogenous expansin than by susceptibility to expansin.

DISCUSSION

We have localized expansin immunologically by tissue printing and immunoblotting (Figs. 1 and 4). Using both methods, we analyzed salt-extractable expansin. It is not known whether some portion of expansin becomes covalently bound in the wall and, therefore, would not be extractable by salt. The most plausible model of expansin action postulates that expansins disrupt hydrogen bonds between cellulose and matrix polymers, and that expansin is released when the hydrogen-bonded complex has pulled apart because its affinity to single wall polymers is lower than its affinity to the paired complex (McQueen-Mason and Cosgrove, 1994). If this is indeed the case, only non-covalently bound expansin could act as a cell wall-

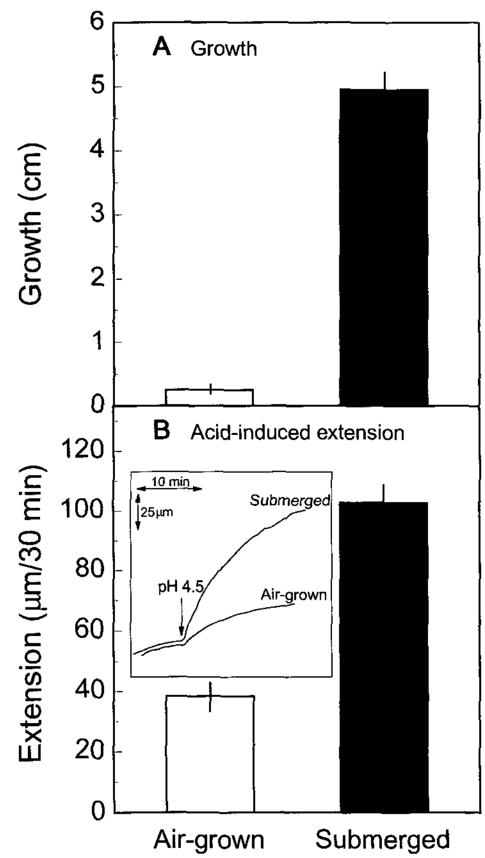

Figure 3. Effect of submergence on internodal growth in rice and on cell wall extension. A, Growth of air-grown and submerged rice internodes. Twenty-centimeter-long stem sections containing the uppermost internode were incubated in air or submerged for 2 d. B, Stem sections were treated as above, and an acid-induced cell wall extension of air-grown and submerged rice internodes was measured, as described in Figure 2. The inset shows representative recorder tracings of the extension of cell walls of air-grown and submerged internodes.

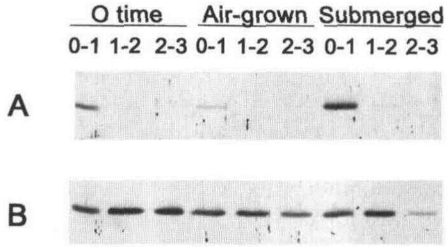

Figure 4. Immunoblot analysis of cell wall proteins from different developmental regions of rice internodes. Cell wall proteins were extracted from each internodal region at 0 time and after incubation of stem sections in air for 2 d or submergence for 2 d. Immunodetection was with the CuEx29 antibody. A, Cell wall proteins per 2.2 mg of cell wall dry weight were loaded in each lane of the SDS-polyacrylamide gel. B, Twenty micrograms of cell wall proteins was loaded in each lane. The numbers indicate the distance (cm) from the node at the base of the internode.

loosening factor, whereas covalently bound expansin would be biologically inactive.

Immunological localization on tissue prints showed that expansin was concentrated mainly in the growing region of the internode (Fig. 1). This was also the region of the highest acid-induced cell wall extensibility and of the highest susceptibility to added expansin (Fig. 2). The physiological significance of the high expansin content in the cells around the developing vascular bundles and along the inner epidermal cell layer (Fig. 1) is not known. Native and

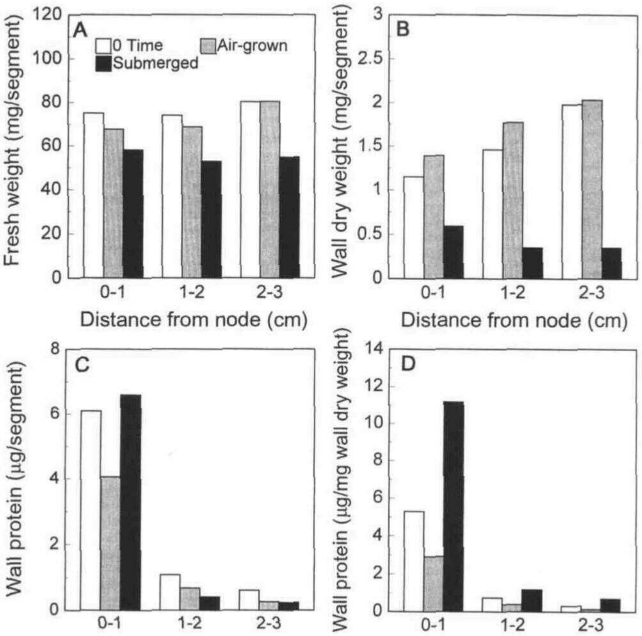

Figure 5. Fresh weight (A), dry weight (B), and protein content (C and D) of cell walls in different developmental regions of rice internodes. Cell walls and cell wall proteins were isolated from each region of the internode at 0 time, after 2 d of incubation of stem sections in air, and after 2 d of submergence. Ninety segments of each internodal region were excised, weighed, and used for wall-protein extraction and wall dry weight measurement.

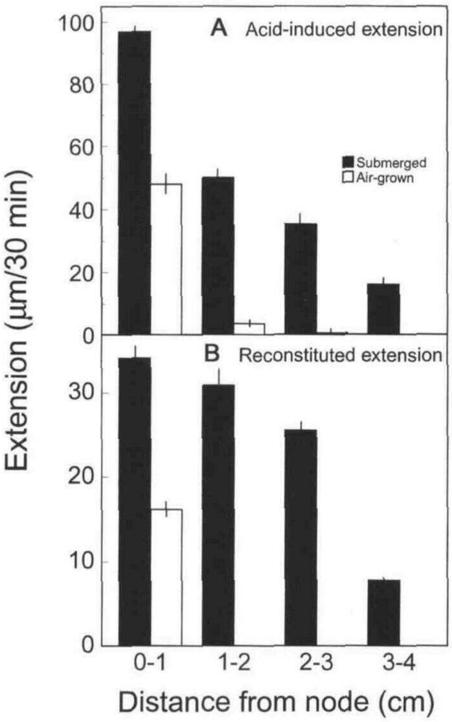

Figure 6. Effect of submergence on cell wall extension of deepwater rice internodes. A, Stem sections were incubated in air or submerged for 2 d. Acid-induced extension of cell walls prepared from developmentally different regions of internodes was measured as described in Figure 2. B, Reconstituted cell wall extension was measured in internodes treated as described above. After preincubation of boiled rice cell walls in 50 mM sodium acetate buffer, pH 4.5, for 20 min, the solutions were changed to the same buffer containing 1 mg mL^{-1} of rice cell wall proteins extracted from the basal 3 to 4 cm of the uppermost internode (Cho and Kende, 1997).

boiled cell wall preparations without the inner epidermis also showed acid-induced and reconstituted extension, respectively (results not shown).

We also used immunoblotting to determine the expansin level in different developmental regions of internodes that had been either kept in air or submerged for 2 d in water (Fig. 4). In evaluating these results, it is very important to consider the parameter on which expansin content should be based. The reason for this is evident from the measurements of the cell wall mass and protein content in the basal 3-cm region of the internode (Fig. 5). In air, growth is very slow, and few new cells are added during 2 d of incubation (Bleecker et al., 1986). The length of submerged internodes increases by approximately 5 cm over 2 d (see Fig. 3A), and all of the cells in the basal 3-cm region are replaced by newly formed cells during this period (Sauter and Kende, 1992a). The fresh and dry weight of the cell walls of air-grown internodes remained close to that of the 0-time control during the 2 d of incubation (Fig. 5, A and B). In contrast, the cell walls of submerged internodes had a somewhat lower fresh weight and a substantially lower dry weight than did the cell walls of air-grown internodes. The reduced dry weight of cell walls in rapidly growing internodal tissue has been noted before (Rose-John and Kende, 1984; Kutschera and Kende, 1989) and has been ascribed to suppression of secondary wall formation. The thickness of the cell wall does not decrease during rapid elongation, indicating that no thinning of the wall takes place (Hoffmann-Benning and Kende, 1994). There was a steep drop in protein content of the cell wall with distance from the node (Fig. 5, C and D). Given these circumstances, we believe that expressing expansin levels per cell wall mass, i.e. per cell wall dry weight, constitutes the physiologically most relevant assessment of expansin content. On this basis, the expansin level was highest in the 1-cm zone above the node (Fig. 4A), where wall extensibility and growth rates were highest (Figs. 2, 3, and 6). When expressed on a protein basis, expansin levels were closely similar in the basal two 1-cm regions of the internode (Fig. 4B). Thus, the ratio of expansins to other cell wall proteins did not change in these regions with submergence or with the developmental stage of the tissue. Because there was such a great drop in the content of total cell wall proteins in the second and third 1-cm zones (Fig. 5, C and D), expressing expansin content on a protein basis (Fig. 4B) leads to comparisons of vastly different cell wall masses and gives a much distorted picture of expansin content in the cell wall.

The growth rate of a plant may be determined, at least in part, by the expansin content of the cell wall and by the susceptibility of the wall to expansin action. It has been suggested that susceptibility of the cell wall to expansin rather than expansin content may control the growth rate of oat coleoptiles (Cosgrove and Li, 1993) and cucumber hypocotyls (McQueen-Mason, 1995). In deepwater rice, expansin content, in addition to susceptibility to expansin, may determine the extensibility of isolated cell walls and the growth rate of the internode. Expansin content (Figs. 1 and 4) and acid-induced cell wall extensibility (Figs. 3 and 6) are related to the growth rate of the tissue, which is enhanced by submergence, is highest at the base of the internode, and declines with distance from the node (Sauter et al., 1993). In rapidly elongating internodes, there is a 2- to 3-cm expansion of the EZ (Bleecker et al., 1986; Sauter et al., 1993). This corresponds to a 2- to 3-cm expansion of acid-induced cell wall extensibility and responsiveness to applied expansin (Fig. 6).

Binding of expansin to cellulose is greatly increased by coating the cellulose with various hemicelluloses, especially with (1→3),(1→4)-β-D-glucan (McQueen-Mason and Cosgrove, 1995). In elongating grass cell walls there is a transient increase in the level of (1→3),(1→4)-β-D-glucan (Carpita, 1984; Luttenegger and Nevins, 1985). This has also been observed in the elongating region of rice internodes (Sauter and Kende, 1992b). This mixed-linked glucan is thought to play a role in the elongating cell walls of grasses, perhaps as the principal hemicellulose that tethers cellulose microfibrils (Carpita and Gibeaut, 1993; Carpita, 1996). The increased binding of expansin to cellulose coated with (1→3),(1→4)-β-D-glucan raises the possibility that hydrogen bonds between cellulose and interlinking (1→3),(1→4)-β-D-glucans may be the main substrate of expansin in the cell walls of grasses. During the transient increase in (1→3),(1→4)-β-D-glucan levels, more expansin-susceptible load-bearing bonds may be available in the cell walls of growing grasses, and disruption of these hydrogen bonds by expansin may result in loosening of the wall.

In conclusion, we showed a close correlation between the distribution of expansin in deepwater rice internodes, acid-induced cell wall extensibility, susceptibility to applied expansin, and submergence-induced elongation. Taken together, these results indicate that expansins play an important role in mediating rapid internodal growth in deepwater rice.

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